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Chemistry of Allergens

XXII. Isolation and Characterization of Three New Antigens from the Dialysates of Six Successive Pepsin Hydrolyses of β-Lactoglobulin[1]

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Abstract. Twelve new antigens previously have been demonstrated in the pepsin digests of milk proteins. The term 'new antigen' is defined as an antigen with a specificity distinct from that of the protein from which it was generated. Three new antigenic (α-, β-, and D2i) polypeptides and one nonantigenic γ-polypeptide have been isolated from the dialysates of six successive pepsin hydrolyses of β-lactoglobulin. The α- and β-polypeptides were $^1/_5$ and D2i was $^1/_{10}$–$^1/_{20}$ as potent immunogens as precursor β-lactoglobulin as determined by the Schultz-Dale technique. The minimum observed amounts of new antigen eliciting maximum response of uterine strips in the Schultz-Dale tests were: α-D2, 50 ng; β-D2, 15 ng; β-D3, 10 ng; and D2i, 1,000 ng. Except for the α-polypeptide, the amino acid contents of the polypeptides differed markedly from that of β-lactoglobulin. The β-polypeptide has been tentatively identified as a 33 amino acid fragment of β-lactoglobulin (3,910 daltons). The γ-polypeptide has been tentatively identified as a 12 amino acid fragment of β-lactoglobulin (1,372 daltons).

This is part of a study on the new antigens generated in a simulated stomach digestion of milk proteins as related to the elucidation of the mechanism of milk allergy. The term 'new antigen' is defined as an antigen with a specificity distinct from that of the protein from which it was

[1] For paper XXI in this series, see reference 16. Experimental investigation of milk allergens was terminated by Agricultural Research Service, US Department of Agriculture, on June 30, 1973.

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generated. It was previously demonstrated by SPIES *et al.* [15] that new antigens were generated by 8-min pepsin hydrolysis of β-lactoglobulin, α-lactalbumin and casein, and that two new antigens were so generated from bovine serum albumin. In a later study [16], β-lactoglobulin was hydrolyzed with pepsin for six successive, 8-min periods during which approximately 90% of the original protein was split into fragments with a molecular weight of 12,000 or less. Six dialysate fractions (D1–D6) and six endofractions (E1–E6) were separated from the hydrolysates and analyzed for the presence of new antigens by the Schultz-Dale and gel diffusion techniques, respectively. All six dialysates contained common nonprecipitating new antigens. The first dialysate (D1) did not contain all of the new antigens common to D2–D6 which indicated the presence of at least two new antigens in D2–D6. There was also an indication of a third new antigen in D6 [16]. Six precipitating new antigens were demonstrated in the endofractions, 2 in E1, 2 in E2 and 2 in E3–E6.

The purpose of this paper is to describe the isolation and chemical and immunochemical characterization of the two new antigens previously demonstrated in the dialysates of the pepsin hydrolysates of β-lactoglobulin and a third new antigen not previously reported.

Experimental Procedure

Materials[3]

β-Lactoglobulin, pepsin, the dialysate of an autodigest of pepsin (PEPD), and the six dialysate fractions (D1-D6) from β-lactoglobulin have been described [16]. In summary, the preparation of D1–D6 consisted of hydrolysis of β-lactoglobulin with pepsin for 8 min at pH 2.0. The reaction was stopped by cooling and adjustment of the pH to 7.5. The hydrolysate was separated into two fractions by dialysis. The dialysate and the inner solution were lyophilized. The inner fraction from each hydrolysis was similarly rehydrolyzed with pepsin and dialyzed. This procedure was done 6 successive times.

Spherical polyacrylamide gels of the Bio-Rad P series were prepared by Bio-Rad Laboratories, Richmond, Calif., and purchased from Calbiochem, Los Angeles, Calif.

Methods

Bio-Gel chromatography. Bio-Gels were swelled with solvent and thoroughly washed with solvent before use. Solvent for all fractionations was water containing 5 ml of chloroform per liter as preservative. Dialysate fraction (D1–D6) were desalted (from the sodium chloride formed in the pepsin digestion) and partially fractionated by passing down through a column of Bio-Gel P-2. Descending

[3] The use of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

chromatography of appropriate fractions was done similarly on columns of Bio-Gel P-10 or P-30 in order to purify the principal components. Column effluents were monitored at 280 or 220 nm. Indicated column fractions were pooled and lyophilized. The purity of each of the pooled fractions was determined by disc electrophoresis.

Analytical disc electrophoresis. The method used was that of DAVIS [4] using solutions described by DAVIS in tables I and II for a 7% separating gel at pH 8.9. A Canalco vertical apparatus was used. Tubes were 63×5 (ID) mm. Samples were from 50 to 500 μg as shown in figures 2 and 9. The tracking dye was bromophenol blue. After electrophoresis, proteins or polypeptides were fixed with 12% trichloroacetic acid and stained with aniline blue-black dye.

Schultz-Dale technique. The basic Schultz-Dale technique used has been described by COULSON [3] and in our previous applications of the method [15–17]. Virgin female guinea pigs, weighing about 225 g, were sensitized on the same day by subcutaneous injections (nuchal area) with two 0.5-ml volumes of antigen emulsified with Freund's complete adjuvant. Emulsions were made with equal volumes of antigen solution in saline or buffered saline and adjuvant. Incubation was for at least 28 days. Challenge doses were administered to the 50-ml Dale bath in which was suspended one half of one of the uterine horns (called strips in this paper) of the sensitized guinea pig. Sensitizing and challenge doses were on a weight basis. None of the antigens reacted nonspecifically with strips from nonsensitized guinea pigs in the quantities used in the challenge doses. To insure that reactions on sensitized tissues were caused by new antigens and not by β-lactoglobulin and/or an autodigest of pepsin, all tests for new antigen were made on strips that either did not react to or were first desensitized to a mixture of β-lactoglobulin and the autodigest of pepsin (PEPD) [15–17]. Almost all strips from animals sensitized with the purified polypeptides did not react to β-lactoglobulin and PEPD.

Amino acid analysis. Tryptophan was determined by the method of SPIES and CHAMBERS [14], procedure N, which was scaled down to use 250- to 500-μg samples. Procedures described by MOORE and STEIN [10] were used for automated amino acid analysis. A small crystal of phenol was added to each sample prior to hydrolysis with 6 N hydrochloric acid to minimize destruction of tyrosine [12]. The samples were heated for 24 h at 110 °C in sealed, evacuated tubes.

Molecular weights. Molecular weights were determined by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS) as described by DUNKER and RUECKERT [5]. The gel concentration used was 12.5%. An essentially straight line was obtained by plotting the log of the molecular weights of the standards versus mobilities as determined as described by WEBER and OSBORN [18]. Chymotrypsin C chain (10,157) insulin (5,700) and glucagon (3,500) were used as standards.

Results

Isolation of New Antigens

Table I contains a summary of the yields, nitrogen contents and percentage of the total nitrogen in each of the dialysate fractions (D1–D6)

Table I. Yields and nitrogen contents of dialysates of successive pepsin hydrolyses of β-lactoglobulin

Hydrolysis No.	Starting material g	Dialysate			
		Symbol of product	yield[1] g	nitrogen %	% of total[2]
1	42.0[3]	C[4]	4.6	4.1	3.2
2	39.0[5]	D2	13.6	8.9	23.4
3	23.4[5]	D3	8.9	8.9	25.6
4	12.4[5]	D4	4.8	8.4	25.2
5	8.0[5]	D5	2.7	8.0	20.4
6	4.8[5]	D6	1.7	8.2	22.3

[1] Inclusive of sodium chloride formed on pepsin hydrolysis.

[2] Based on total nitrogen content of starting sample.

[3] β-Lactoglobulin.

[4] This fraction was redialyzed yielding 3.5 g of the sample (D1) used in this work. Nitrogen content of D1, 3.6%.

[5] Retentate from dialysis of the immediately preceding hydrolysis.

obtained in the successive hydrolyses of β-lactoglobulin [16]. D2 and D3 were used for the isolation of the purified new antigens because they not only contained the new antigens common to all of the dialysates [16, 17] but they also were available in greater quantity than the other fractions. Figure 1A, B shows the desalting and partial fractionation of D2 and D3 on Bio-Gel P-2 into three fractions, A, B, and C, respectively. D2A and D3A ('A' fractions) contained most of the new antigens; D2B and D3B ('B' fractions) contained much less of new antigen than did the A fractions; D2C and D3C ('C' fractions) were not antigenic. The B and C fractions were not studied further. Table II contains a summary of the yields of A, B, and C fractions obtained from D1–D6. The relatively low percentage yield of both D1 (table I) and D1A is consonant with the fact that pepsin hydrolyzes native proteins poorly. Thus, the denaturation of β-lactoglobulin, that undoubtedly occurred in obtaining D1, was sufficient so that there was much more hydrolysis in subsequent hydrolyses as shown by the higher percentage yields of D2–D6 than of D1. The percentages of nonantigenic C fractions obtained from D2 and D3 were 16.6 and 12.8%, respectively, which greatly exceeded that from D4 (2.0%) and none from D5 and D6.

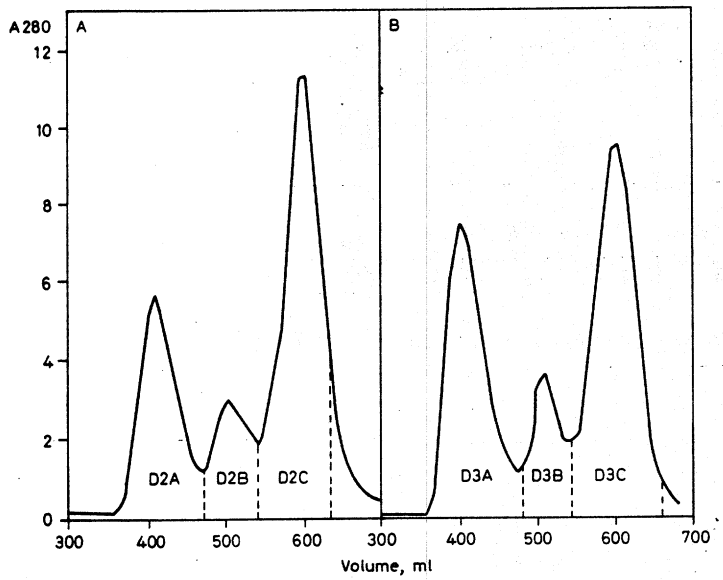

Fig. 1. Desalting and fractionation of D2 and D3 on Bio-Gel P-2. Column: 5×50 cm. Solutions of D2 and D3 were prepared for chromatography essentially as follows. Suspensions of D2 and D3, containing 67 mg/ml of solvent, were stirred for at least 2 h and centrifuged. The clear supernatant solutions were decanted from an insoluble gel (in the case of D3, the gel was extracted once and the extract was added to the original solution). Final concentrations (based on original weights of D2 and D3) were adjusted as shown below. *A* 40 ml of solution from 2.25 g of D2 was chromatographed. Corresponding fractions from 4 similar runs (8.2 g D2) were combined and lyophilized. Sodium chloride first appeared at 644 ml. *B* 40 ml of solution from 1.95 g of D3 was chromatographed. Corresponding fractions from 4 similar runs (7.8 g D3) were combined and lyophilized. Sodium chloride first appeared at 670 ml.

Figure 2 shows the disc electrophoretic patterns of the A fractions from all six dialysates on a comparative basis. There are three principal bands which have been designated α, β and γ in the order of their mobilities. Although disc electrophoresis of some subfractions showed up to eight bands, many of which were relatively faint, the isolation of the polypeptides represented by these minor bands was not attempted.

Figure 3A shows the rechromatography of D2A on Bio-Gel P-30, and figure 3B that of D3A on Bio-Gel P-10 which yielded subfractions from which the purified polypeptides, α-, β-, and γ-D2 and β-D3 and γ-D3, and D2i were ultimately obtained.

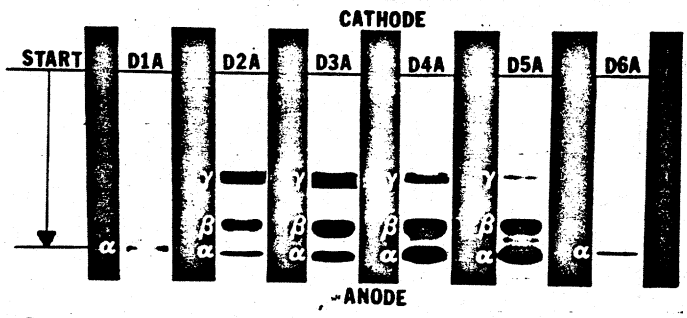

Fig. 2. Comparative disc electrophoresis patterns of A fractions from D1–D6. Samples were 500 μg of each.

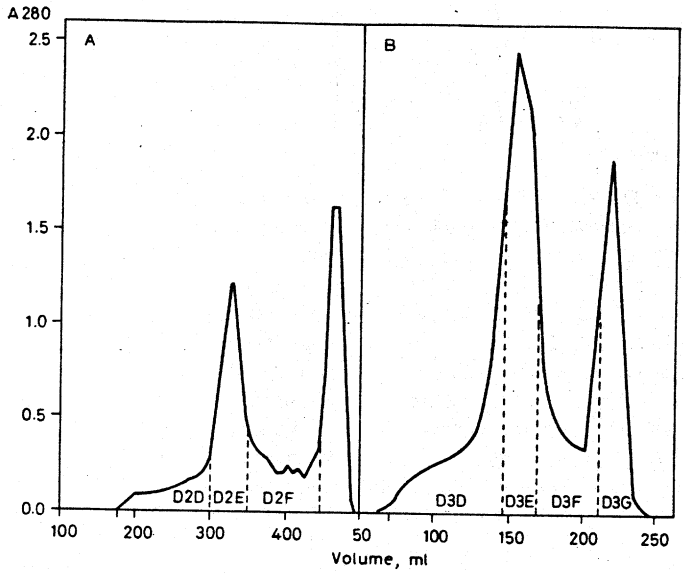

Fig. 3. Fractionation of D2A and D3A on Bio-Gel P-30 and P-10, respectively. Column: 2.5×105 cm. *A* Solutions of D2A were made up as follows. To 250 mg of D2A was added 4.0 ml of solvent. The suspension was stirred and a gel was separated by centrifugation. The gel was extracted with 1-, 1-, 1-, and 2-ml portions of solvent. The supernatant solution from each extraction was added to the original solution. The final solution of D2A (8 ml) was chromatographed on Bio-Gel P-30. Corresponding fractions from 7 similar runs (1.75 g D2A) were combined and lyophilized. *B* Solutions of 250 mg of D3A were prepared similarly to those of D2A except that the 2-ml washing of the insoluble gel was omitted. The solution from 250 mg of D3A was chromatographed on Bio-Gel P-10. Corresponding fractions from 7 similar runs (1.75 g D3A) were combined and lyophilized.

Table II. Fractions obtained by desalting and partial fractionation of D1–D6 on Bio-Gel P-2[1]

	Dialysate	A fraction[2]		B fraction		C fraction	
	g	g	%	g	%	g	%
D1	0.80	0.036	4.5	0.066[3]	8.3	0.011[3]	1.4
D2[4]	8.20	2.050	25.0	1.340[2]	16.4	1.26[3]	16.6
D3[4]	7.80	2.230	28.6	1.220[2]	15.6	1.00[2]	12.8
D4[5]	3.70	1.340	36.0	0.540[2]	14.6	0.073[3]	2.0
D5	1.60	0.540	33.8	0.220[2]	13.8	0	0
D6	0.20	0.067	33.5	0.019[2]	9.5	0	0

[1] Fractionated as described in figure 1.
[2] Sodium chloride-free.
[3] Sodium chloride detectable.
[4] Fractionated in 4 approximately equal portions. Corresponding fractions from each run were combined and lyophilized to give total A fraction.
[5] Fractionated in 2 equal portions. Corresponding fractions from each run were combined and lyophilized to give total A fraction.

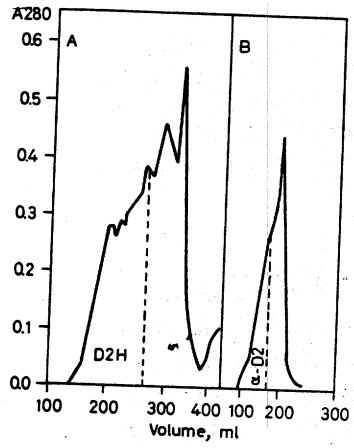

Fig. 4. Isolation of α-D2 from D2D (fig. 3A) by fractionations on Bio-Gel P-30. Column: 2.5×105 cm. *A* 7 ml of solution containing 57 mg of D2D was chromatographed and indicated fractions were combined and lyophilized to yield 21.1 mg of D2H. *B* 6 ml of a solution containing 19.7 mg of D2H was chromatographed and indicated fractions were combined and lyophilized to yield 7.0 mg of α-D2.

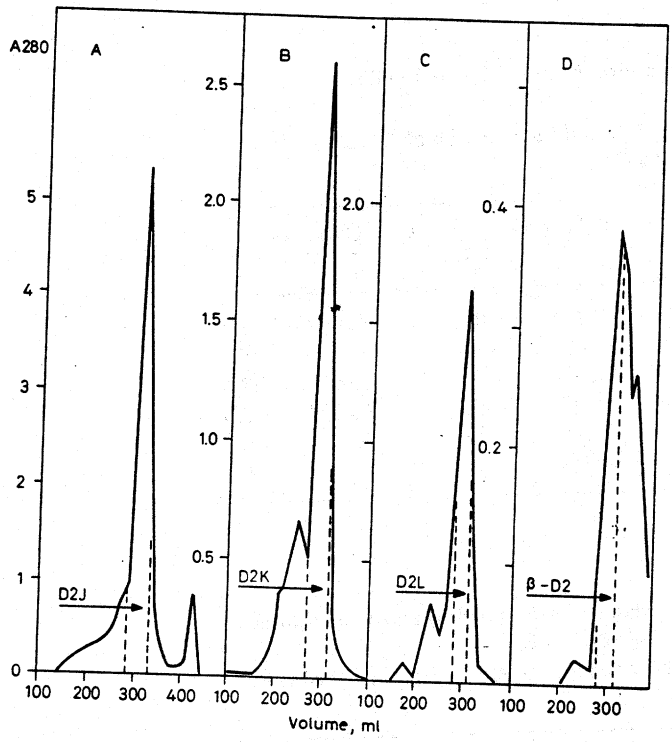

Fig. 5. Isolation of β-D2 from D2E (fig. 3A) by fractionations on Bio-Gel P-30. Column: 2.5×105 cm. *A* 6 ml of a solution containing 272 mg of D2E was chromatographed and indicated fractions were combined and lyophilized to yield 107.4 mg of D2J. *B* 8 ml of solution containing 104 mg of D2J was chromatographed and indicated fractions were combined and lyophilized to yield 57.2 mg of D2K. *C* 7 ml of a solution containing 55.2 mg of D2K was chromatographed and indicated fractions were combined and lyophilized to yield 23.5 mg of D2L. *D* 8 ml of a solution containing 20.5 mg of D2L was chromatographed and indicated fractions were combined and lyophilized to yield 5.5 mg of β-D2.

Figure 4A, B shows the isolation of the purified polypeptide, α-D2: figure 4A, chromatography on Bio-Gel P-30 of D2D (fig. 3A) and figure 4B, similar rechromatography of succeeding fraction D2H to give α-D2.

Figure 5A–D shows the isolation of the purified polypeptide, β-D2, by: figure 5A, chromatography on Bio-Gel P-30 of D2E (fig. 3A); figure 5B–D, similar rechromatography of successive fractions, D2J, D2K and finally D2L to give β-D2.

Figure 6A–C shows the isolation of the purified polypeptide, γ-D2

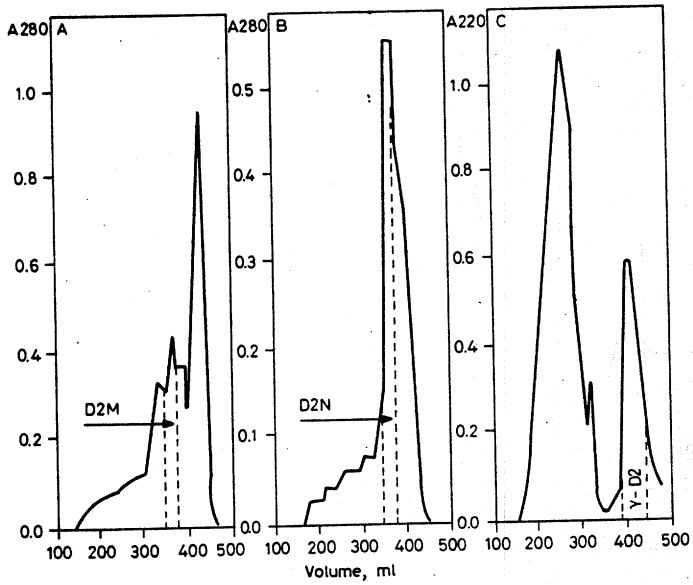

Fig. 6. Isolation of γ-D2 from D2F (fig. 3A) by fractionation on Bio-Gel P-30 and isolation of D2i as the insoluble residue obtained from D2F. Column: 2.5×105 cm. *A* The solution of D2F was prepared similarly to that of D2A (fig. 3). The insoluble gel so obtained was lyophilized to yield 45.5 mg of D2i. The solution obtained from 253 mg of D2F was chromatographed. Corresponding fractions from two similar runs (513 mg of D2F) were combined and lyophilized to yield 197.7 mg of D2M. *B* The solution of D2M was prepared similarly to that of D2A and practically all of D2M dissolved. 8 ml of the solution from 189 mg of D2M was chromatographed and indicated fractions were combined and lyophilized to yield 30.3 mg of D2N. *C* 6 ml of a solution containing 21.5 mg of D2N (all of D2N readily dissolved) was chromatographed and indicated fractions were combined and lyophilized to yield 1.5 mg of γ-D2.

by: figure 6A, chromatography on Bio-Gel P-30 of D2F (fig. 3A); figure 6B, C, similar rechromatography of successive fractions D2M and finally D2N to give γ-D2.

D2i

Portions of fractions D2A and D3A and some of their subfractions, particularly those leading to the isolation of γ-D2 and γ-D3, were insoluble in the quantities of solvent used for chromatography. In early experiments, this insoluble gel was separated by centrifugation, washed and discarded. Fortunately, the insoluble material obtained from D2F,

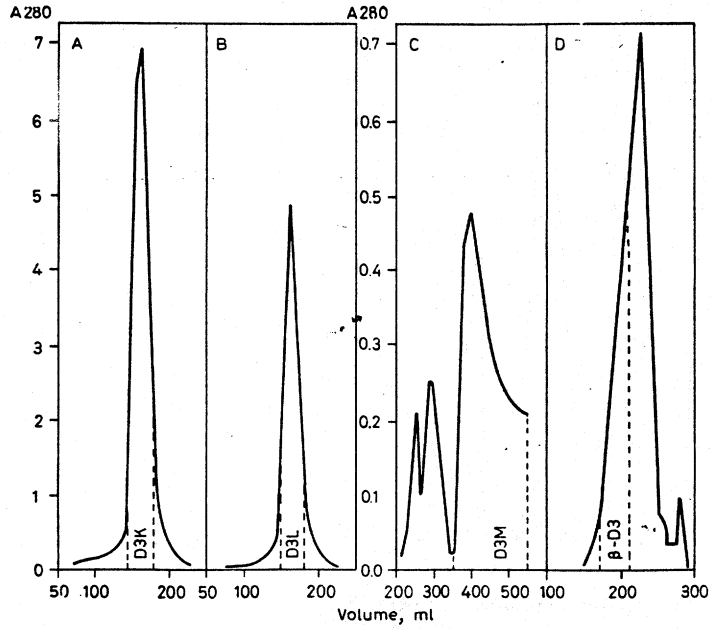

Fig. 7. Isolation of β-D3 from D3E (fig. 3B) by fractionations on Bio-Gel P-10 and P-30. Column: 2.5×105 cm. *A* To 218 mg of D3E was added 4.0 ml of solvent. The suspension was stirred briefly and centrifuged. The insoluble gel was extracted successively with three 1-ml volumes of solvent and the extracts were combined with the original solution. The final solution of D3E was chromatographed on Bio-Gel P-10 and indicated fractions were combined and lyophilized to yield 125.5 mg of D3K. *B* 6 ml of solution containing 117.9 mg of D3K, which dissolved completely, was chromatographed on Bio-Gel P-10 and indicated fractions were combined and lyophilized to yield 66.8 mg of D3L. *C* 6 ml of a solution containing 60.7 mg of D3L was chromatographed on Bio-Gel P-30 and indicated fractions were combined and lyophilized to yield 27.4 mg of D3M. *D* 6 ml of a solution containing 24.2 mg of D3M was chromatographed on Bio-Gel P-30 and indicated fractions were combined and lyophilized to yield 6.5 mg of β-D3.

as described in the legend of figure 6, designated D2i, proved to be a potent new antigen, immunologically distinct from both α- and β-polypeptides.

Figure 7A–D shows the isolation of the purified polypeptide, β-D3 by: figure 7A, chromatography on Bio-Gel P-10 of fraction D3E (fig. 3B); figure 7B, similar rechromatography of resulting fraction D3K; figure 7C, D, rechromatography on Bio-Gel P-30 of successive fractions D3L and finally D3M to give β-D3.

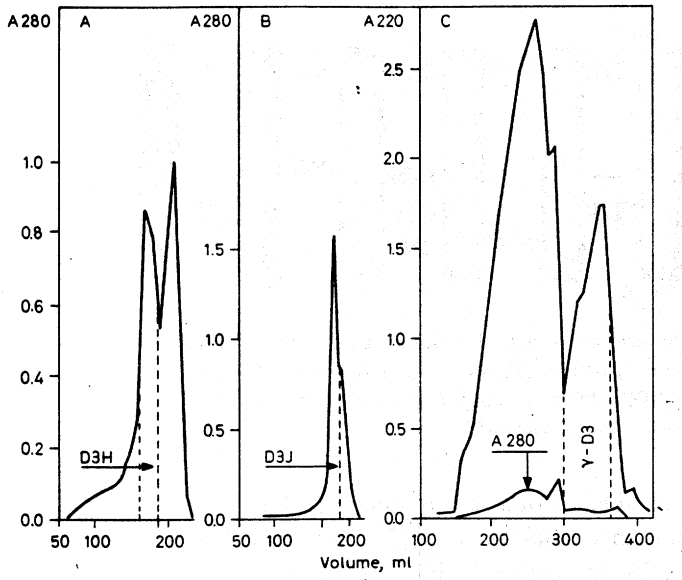

Fig. 8. Isolation of γ-D3 from D3F (fig. 3B) by fractionations on Bio-Gel P-10 and P-30. Column: 2.5×105 cm. *A* Solution of 274 mg of D3F was prepared similarly to that of D2A (fig. 3A). 8 ml of the solution was chromatographed on Bio-Gel P-10. Corresponding fractions from two similar runs (548 mg of D3F) were combined and lyophilized to yield 157.4 mg of D3H. *B* Solution from 153 mg of D3H was prepared like that of D3F. 7 ml of this solution was chromatographed on Bio-Gel P-10 and indicated fractions were combined and lyophilized to yield 65.9 mg of D3J. *C* 6 ml of a solution containing 57.3 mg of D3J (all completely dissolved) was chromatographed on Bio-Gel P-30 and indicated fractions were combined and lyophilized to yield 7.3 mg of γ-D3.

Figure 8A–C shows the isolation of the purified polypeptide, γ-D3 by: figure 8A chromatography on Bio-Gel P-10 of D3F (fig. 3B); figure 8B, similar rechromatography of resulting fraction D3H; and figure 8C, rechromatography on Bio-Gel P-30 of resulting fraction D3J to give γ-D3.

Comparative Disc Electrophoresis of Original β-Lactoglobulin and Isolated Fractions

Figure 9 shows the comparative disc electrophoretic pattern of the original sample of β-lactoglobulin with those of D2A and D3A, and the purified polypeptides, α-D2, β-D2, γ-D2, β-D3, γ-D3 and D2i. The major bands from β-D2 and β-D3 corresponded as did those from γ-D2

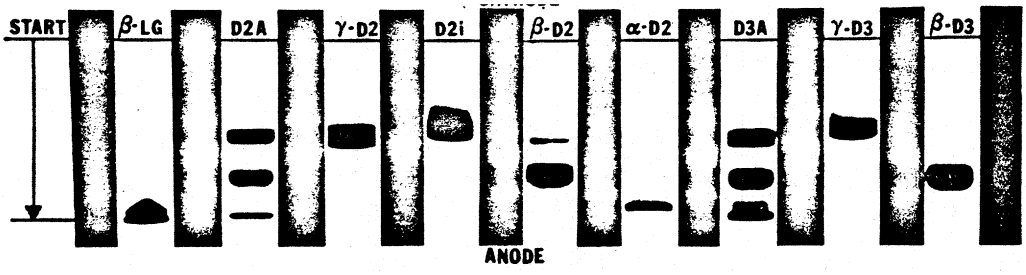

Fig. 9. Comparative disc electrophoresis of β-lactoglobulin (β-Lg, 50 μg), D2A (450 μg) and purified polypeptides γ-D2, β-D2, α-D2 and D2i and D3A (450 μg) and purified polypeptides γ-D3 and β-D3. All purified polypeptide samples were 150 μg and each was run similarly as described in Methods.

and γ-D3. β-D2 contained a detectable amount of another band in the γ-D2 region. β-D3 was freer from this band than β-D2 although β-D3 retained a trace not shown in the figure. That the single band from D2i corresponded with the γ-bands may be coincidental although they are compositionally somewhat similar.

Immunogenicity of the New Antigens

Table III shows the immunogenicity of β-lactoglobulin as compared with that of the new antigens in guinea pigs as determined by the Schultz-Dale technique using a uniform 10-μg challenge dose of each antigen. 5 μg of β-lactoglobulin sensitized over 50% of the animals as compared with 25 μg for α-D2 and β-D2 and 50–100 μg for D2i. However, over 50% of the animals sensitized with 10 μg of D2i gave submaximal reactions when the challenge dose was increased to 100 μg of D2i. 200 μg of γ-D3 did not sensitize any of three guinea pigs tested and only one of four guinea pigs sensitized with 100 μg of γ-D3 gave a submaximal response even though challenged with 100 μg of γ-D3. It is apparent that γ-D3, if immunogenic at all, is less than 10% as potent as α-D2, β-D2 or D2i.

Antigenicity of New Antigens

Systematic determination of the minimum amount of the new antigens that would give a maximum challenge response (90–100%) in the Schultz-Dale test in homologously sensitized guinea pigs was not attempted. However, several near minimal amounts of each new antigen

Table III. Comparative immunogenicity of β-lactoglobulin (β-Lg) and new antigens using Schultz-Dale technique[1]

Antigen	Sensitizing dose, μg	Challenge dose, μg	Animals tested No.	Reaction[2]		
				maximal No.	sub-maximal No.	negative No.
β-Lg	10	10	4	3	1	0
	5	10	2	2	0	0
	2	10	3	1	0	2
	1	10	3	0	0	3
α-D2	25	10	4	3	1	0
	10	10	4	0	0	4
	5	10	4	0	0	4
β-D2	25	10	5	5	0	0
	10	10	3	0	1	2
	5	10	3	0	0	3
β-D3	100	0.01–1.0	4	3	0	1
γ-D3	200	100	3	0	0	3
	100	100	4	0	1	3
D2i	100	10	10	6	2	2
	50	10	5	2	2	1
	25	10	4	1	0	3
	10	10	4	0	0	4

[1] Challenge tests for new antigens were made on uterine strips from sensitized guinea pigs that either did not react with or were previously desensitized with β-lactoglobulin plus an autodigest of pepsin (PEPD) to insure that reaction was due to new antigen and not β-lactoglobulin or pepsin. See text and references 15, 16.

[2] Maximal reaction, 90–100% that of histamine. Submaximal reaction, <90% that of histamine.

that gave a maximum response, as determined incidentally to other studies, is shown in table IV, together with the sensitizing dosages of the new antigens used. The minimum amounts so determined were: α-D2, 25 ng; β-D2, 15 ng; β-D3, 10 ng and D2i 1,000 ng.

Immunological Specificity Relationships of the New Antigens

Although, the disc electrophoretic patterns (fig. 9) indicate excellent separation of α-, β- and D2i new antigens, some cross-reactions between

Table IV. Near minimal amounts of new antigens giving a maximum response in Schultz-Dale testing of homologously sensitized guinea pigs

New antigen	Sensitizing dose, mg	Challenge dose, ng	Reaction, %[1]
α-D2	100	25	95
	50	50	96
β-D2	50	40	98
	25	15	94
β-D3	100	10	100
D2i	200	1,000	100

[1] Based on histamine reaction as 100%.

them occasionally occurred in the sensitive Schultz-Dale tests. However, it was shown by quantitative estimation that cross-reacting components amounted to less than 10% of the sensitizing antigen.

Figure 10 shows results of a Schultz-Dale test using a guinea pig sensitized with α-D2 which demonstrates that β-D2 contains <10% of α-D2.

Figure 11 shows results of a Schultz-Dale test on a guinea pig sensitized with β-D2 which demonstrates that α-D2 contains <25% of β-D2. In another similar experiment with a β-D3 preparation other than the one described herein, it was shown that α-D3 contains <10% β-D3.

Figure 12 shows results of a Schultz-Dale test on a guinea pig sensitized with D2i which shows that α-D2 and β-D2 contain <10% of D2i.

These results conclusively demonstrate that a significant concentration of three distinct new antigens is contained in the purified α-, β-, and D2i polypeptides. As is the case with all biologically potent preparations separated from a complex mixture, it is recognized that the exhibited new antigen property could be contained in an undetected contaminant. Decision on this point must await future experimentation.

Amino Acid Analysis of New Antigens and Their Tentative Identification with Known Polypeptide Chains of the β-Lactoglobulin Molecule

Results of compositional analysis of air-dried samples of γ-D2, γ-D3, β-D2, β-D3, α-D2, of D2i and β-lactoglobulin AB for comparison are shown in tables V–VII. Tryptophan contents, as determined on solid

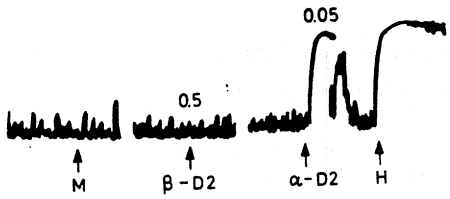

Fig. 10. Demonstration of the antigenicity of new antigen α-D2 and that β-D2 contains <10% of α-D2 by the Schultz-Dale technique. Sensitizing antigen: 50 μg α-D2. Challenge doses in micrograms: M (β-lactoglobulin and PEPD), each component, 10; β-D2, 0.5; α-D2, 0.05; H (histamine), 100.

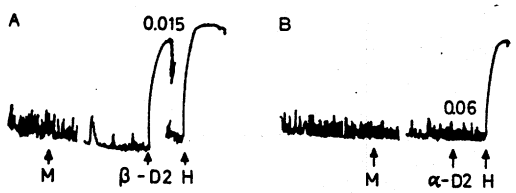

Fig. 11. Demonstration of the antigenicity of new antigen β-D2 and that α-D2 contains <25% of β-D2 by the Schultz-Dale technique. Sensitizing antigen: 25 μg β-D2. Challenge doses in micrograms: M (β-lactoglobulin and PEPD), each component, 10. *A* β-D2, 0.015; H (histamine), 100. *B* α-D2, 0.06; H (histamine), 100.

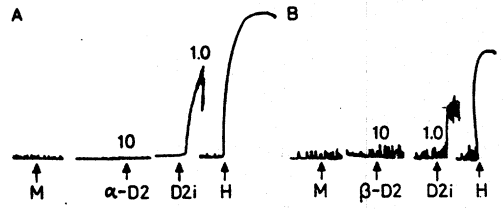

Fig. 12. Demonstration of the antigenicity of new antigen D2i and that D2i contains <10% of α-D2 and β-D2 by the Schultz-Dale technique. Sensitizing antigen: 50 μg D2i. Challenge doses in micrograms: M (β-lactoglobulin and PEPD), each component, 10. *A*. α-D2, 10; D2i. 1.0; H (histamine), 100. *B* β-D2, 10; D2i, 1.0; H (histamine), 100.

samples, are shown in table V. Table VI lists percentages of amino acids found in the acid hydrolysates. Clearly, the isolated polypeptides vary in amino acid composition and all differ from β-lactoglobulin. γ-D2 and γ-D3 polypeptides are virtually identical. The same is true for β-D2 and β-D3 polypeptides. D2i resemble the γ-polypeptide in some ways, but there are large differences in content of many amino acids, particularly,

Table V. Tryptophan contents of β-lactoglobulin and new antigens

New antigen	Tryptophan content, %[1]
β-Lg	1.95[2]
α-D2	1.96
β-D2	4.73
β-D3	4.99
γ-D2	0.07
γ-D3	0.03
D2i	0.17

[1] All air-dried basis.
[2] Procedure U [13].

Table VI. Amino acid composition of polypeptides and β-lactoglobulin AB; percentage found in 24-hour hydrolysate of air-dried samples

	β-Lg AB	α-D2	β-D2	β-D3	γ-D2	γ-D3	D2i
Tryptophan[1]	1.75	3.19[2]	2.19	2.24	0	0.41	0
Lysine	12.40	8.24[2]	17.70	18.00	17.90	17.60	15.50
Histidine	1.75	1.18	3.41	3.65	0	0.22	0.99
NH_3	1.37	1.75	1.51	1.61	2.28	2.05	1.66
Arginine	2.72	2.67	0	0	0.47	0.30	1.40
Aspartic acid	11.50	10.30	5.04	5.20	25.30	22.40	17.50
Threonine	5.01	3.40	2.84	2.92	0.46	0.32	5.03
Serine	3.69	3.61	0	0	0.31	0.12	0.32
Glutamic acid	20.36	29.20	28.30	28.90	10.50	10.60	11.40
Proline	5.02	4.94	2.73	2.81	0.39	0.48	1.26
Glycine	1.42	0.70	0.85	0.90	0.31	0.27	1.66
Alanine	7.06	5.79	6.45	6.58	6.21	6.39	4.83
Cystine, 1/2	2.62	6.46	4.83	4.90	0	0	0
Valine	5.96	5.35	2.77	2.85	10.40	11.50	9.76
Methionine	3.24	2.63	0	0	0.39	0.42	4.08
Isoleucine	6.11	4.04	10.50	10.70	7.56	7.10	7.01
Leucine	16.00	8.12	3.25	3.43	18.30	20.80	22.00
Tyrosine	3.98	1.47	0	0	0	0	0.29
Phenylalanine	3.59	3.55	3.67	3.69	0.30	0.84	1.44

[1] These tryptophan contents were determined from the chromatograms and hence are influenced by the effects of the acid hydrolysis
[2] These peaks were poorly resolved in the chromatogram.

threonine, glycine and methionine. The α-D2 polypeptide is unlike any of the others; however, it bears some resemblance to β-lactoglobulin.

The same results calculated as molar ratios with either alanine or phenylalanine assigned the value 1 appear in table VII. Of course, highly accurate values for tryptophan, serine, threonine, half-cystine, valine and isoleucine would not be expected from analysis of a 24-hour hydrolysate. For the control sample of β-lactoglobulin AB, agreement with accepted values is good. As pointed out previously, there are some similarities between α-D2 polypeptide and β-lactoglobulin while D2i is quite different in composition. Until further studies of these antigens are made, little more can be said with certainty. One may speculate that the γ-polypeptide is the fragment -Lys-Leu-Asp-Ala-Ile-Asn-Glu-Asn-Lys-Val-Leu-Val- (residues 83 through 94) in the sequence of β-lactoglobulin proposed by BRAUNITZER *et al.* [2]. The β-polypeptide may also be identified tentatively in the BRAUNITZER *et al.* sequence favored by FRANK and reported by LYSTER [7]. The analyses of the β-polypeptide suggest that this is made up of 33 amino acid residues: Lys_6 His_1 Trp_1 $Asp_{1.5}$ Thr_1 Glu_9 Pro_1 $Gly_{0.5}$ Ala_3 $^1/_2$ Cys_2 Val_1 Ile_4 Leu_1 Phe_1; four amide groups are present.

Assuming the half-cystines to be in the form of a disulfide bridge, and that the Asp⇌Gly substitution occurs in this portion of the chain, a polypeptide of this composition exists in the Braunitzer sequence as residues 59 through 84 and 156 through 162, with the -S-S- bond located at 66–160. Location of one disulfide bridge in the Braunitzer sequence between 66 and 160 has been proposed by MCKENZIE *et al.* [9]. The Asp⇌Gly substitution is at residue 64 in this sequence. The proposed sequence for the β-polypeptide is shown in figure 13A.

Several fragments are placed differently in the Frank sequence as communicated to LYSTER [7]. However, an equivalent polypeptide, containing the Asp⇌Gly substitution at residue 122, a disulfide bridge, and of identical composition, is present here as residue 116 through 141 and 156 through 162. The corresponding disulfide bridge is between 123 and 160. On the assumption that this is the correct sequence of β-lactoglobulin, the location of a 123–160 disulfide bridge is in accord with the work of MCKENZIE *et al.* [9] and that of PREAUX as cited by LYSTER [7] and MCKENZIE *et al.* [9]. This proposed sequence for the β-polypeptide is shown in figure 13B.

In the published sequences there are five rather than four amide groups in this bridged polypeptide. If the β-polypeptide does indeed con-

Table VII. Amino acid composition of polypeptides and β-lactoglobulin AB; calculated molar ratios based on either phenylalanine or alanine = 1 (assumed number of residues shown in parantheses)

	β-Lg AB		α-D2, Phe=1	β-D2, Phe=1	β-D3, Phe=1	γ-D2, Ala=1	γ-D3, Ala=1	D2i, Ala=1
	theo-retical[1], Phe=1	found, Phe=1						
Tryptophan	0.50	0.40	0.73	0.48 (1)	0.49 (1)	0	0	0
Lysine	3.75	3.89	2.63	5.46 (6)	5.52 (6)	1.76 (2)	1.67 (2)	1.95
Histidine	0.50	0.52	0.35	0.99 (1)	1.05 (1)	0	0.02	0.12
NH_3	3.75	3.70	4.79	4.00 (4)	4.24 (4)	1.92 (2)	1.68 (2)	1.80
Arginine	0.75	0.73	0.71	0	0	0.04	0.02	0.15
Aspartic acid	3.88	3.97	3.59	1.70 (1.5)	1.75 (1.5)	2.73 (3)	2.35 (3)	2.43
Threonine	2.00	1.94	1.33	1.07 (1)	1.10 (1)	0.06	0.04	0.78
Serine	1.75	1.62	1.60	0	0	0.04	0.02	0.06
Glutamic acid	6.25	6.38	9.23	8.66 (9)	8.79 (9)	1.02 (1)	1.00 (1)	1.43
Proiine	2.00	2.01	2.00	1.07 (1)	1.09 (1)	0.05	0.06	0.20
Glycine	0.88	0.87	0.43	0.51 (0.5)	0.54 (0.5)	0.06	0.05	0.41
Alanine	3.63	3.65	3.03	3.26 (3)	3.31 (3)	(1)	(1)	(1)
Cystine, 1/2	1.25	1.01	2.50	1.81 (2)	1.83 (2)	0	0	0
Valine	2.38	2.34	2.13	1.06 (1)	1.09 (1)	1.28 (2)	1.37 (2)	1.54
Methionine	1.00	1.00	0.82	0	0	0.04	0.04	0.50
Isoleucine	2.50	2.15	1.44	3.62 (4)	3.64 (4)	0.83 (1)	0.75 (1)	0.99
Leucine	5.50	5.63	2.89	1.12 (1)	1.17 (1)	2.00 (2)	2.21 (2)	3.09
Tyrosine	1.00	1.01	0.38	0	0	0	0	0.03
Phenylalanine	(1)	(1)	(1)	(1)	(1)	0.03	0.07	0.16

[1] McKenzie [8].

tain the 33 amino acid residues indicated from its amino acid composition, its minimum molecular weight is 3,910. Its theoretical tryptophan content is 5.22%. As shown in table V, β-D2 contained 4.73 and β-D3 4.99% tryptophan, values in excellent agreement with the theoretical content. In future work we hope to sequence the isolated antigenic polypeptides and to establish more firmly their positions in the β-lactoglobulin molecule.

Molecular Weight of New Antigens

Results of the molecular weight determinations on the new antigens, both with iodoacetamide and with mercaptoethanol are shown in table VIII.

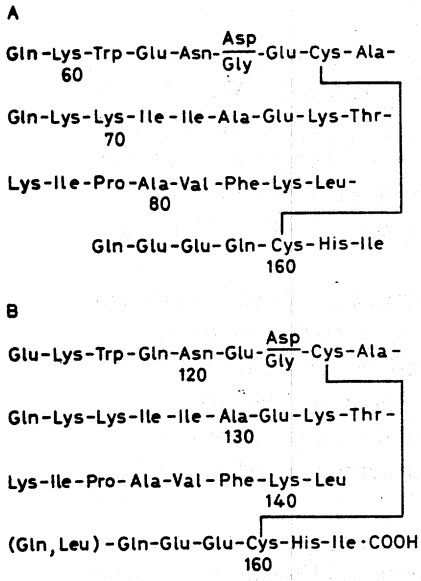

Fig. 13. Provisional sequences of the 33 amino acid β-polypeptide. *A* According to BRAUNITZER *et al.* [2]. *B* According to FRANK as communicated to LYSTER [7] and MCKENZIE *et al.* [9].

Table VIII. Molecular weight of new antigens by sodium dodecyl sulfate-polyacryiamide gel electrophoresis in presence of iodoacetamide and 2-mercaptoethanol

New antigen	Molecular weight[1]	
	iodoacetamide	2-mercapto-ethanol
β-D2	7,300 ± 200	4,825 ± 175
α-D2	9,225 ± 575	5,775 ± 425
D2i	9,775 ± 340	9,315 ± 445

[1] Average of 2 determinations except for D2i which was the average of 4 determinations.

Discussion

Three of the four purified, new antigen polypeptides were potent immunogens although they were not as potent as the precursor protein as determined by their capacities to sensitize guinea pigs and produce max-

imum response in 50% or more of the uterine strips of the animals on challenge by the Schultz-Dale technique. α-D2 and β-D3 were $^1/_5$ and D2i was $^1/_{10}$–$^1/_{20}$ as potent immunogens as β-lactoglobulin when all strips were uniformly challenged with 10 μg of homologous sample. However, D2i, when challenged with 100 μg of sample, was $^1/_2$ as potent an immunogen as β-lactoglobulin challenged with 10 μg. The γ-polypeptide was not immunogenic.

The complex subject of the chemistry of antigens and its relationship to immunogenicity (ability to elicit an antibody response) of proteins and polypeptides has been excellently reviewed by GILL [6]. Among the most important factors influencing immunogenicity of the new antigens pertinent to this discussion are composition, molecular weights as well as primary, secondary, tertiary and quaternary structures as related to these same properties of β-lactoglobulin.

One of the earliest postulated, compositional requirements for immunogenicity was thought by OBERMAYER and PICK [11] to be the presence of the aromatic grouping within the molecule. Later studies have shown that the effect of aromatic amino acids on immunogenicity is variable but not essential. The general conclusion according to GILL [6] is that the presence of aromatic amino acids enhances immunogenicity of proteins and polypeptides and that there is a particular amount necessary for optimal enhancement.

The isolated polypeptides show considerable variation in composition. Several of the amino acids occurring in β-lactoglobulin are absent from the β-, γ- and D2i polypeptides. Although the α-polypeptide contains all of the amino acids found in β-lactoglobulin, some of them are in significantly different proportions. The β-polypeptide contains twice as much tryptophan as β-lactoglobulin but it contains no arginine, serine, methionine or tyrosine, hence these missing amino acids are not necessary for its immunogenicity. The nonimmunogenic γ-polypeptide contains no tryptophan, cystine or tyrosine and, on a residue basis (alanine = 1, table VII), the very low amounts of histidine, arginine, threonine, serine, proline, glycine, methionine and phenylalanine may be due to impurities. D2i, however, which is a potent immunogen, also contained no tryptophan or cystine and, on a residue basis (alanine = 1, table VII), the low amounts of histidine, arginine, serine, proline, tyrosine and phenylalanine also may be due to impurities. It is noteworthy that both the γ-polypeptide and D2i contain no appreciable amounts of aromatic amino acids and that one of these polypeptides is nonimmunogenic and one

is a potent immunogen. It is apparent that the only clear-cut differences in composition between the γ-polypeptide and D2i are the much higher threonine and methionine contents of D2i. Available evidence does not permit the conclusion that these differences in amino acid contents account for the immunogenicity of D2i and the nonimmunogenicity of the γ-polypeptide. An alternative explanation for, exclusive of possible effects of different molecular weights, is that under the conditions of immunization, the guinea pig may develop tolerance instead of immunity as it is known that this property depends on delicately balanced properties of both the host and the immunizing substance.

The molecular weight of 7,300 found for the β-polypeptide by the SDS method does not agree with the minimum molecular weight of 3,910 for the proposed 33 amino acid polypeptides (fig. 13A, B). Also the molecular weight of 4,825 for the reduced β-D2 by the SDS method does not agree with the molecular weight of 3,027 for the largest fragment that would be formed by cleavage of the disulfide bond of the proposed sequence in figure 13A. However, the purity of the β-polypeptide, as indicated by the excellent agreement between found and calculated numbers of residues as well as the established accuracy of the amino acid analytical methods, enhances the probability of the accuracy of the proposed 33 amino acid monomer with a molecular weight of 3,910. The higher than expected values for the molecular weights of β-D2 and reduced β-D2 by the SDS method can only be explained by the known difficulty in determination of molecular weights of polypeptides and proteins of 10,000 daltons or less or to a possible anomalous polymerization of β-D2 in the SDS procedure [18]. Resolution of this question was not possible owing to termination of the investigation. The molecular weight of the α-D2 polypeptide of 9,225 and a lower value (5,775) for the reduced α-D2 was expected because of the disulfide linkage. D2i had a molecular weight of 9,775 and the molecular weight of reduced D2i was 9,313, a value anticipated due to the absence of appreciable disulfide in the molecule. Although the molecular weights of the new antigens are lower than optimum for immunogenicity, nevertheless, all of the molecular weights from 3,910 to 9,775 are well above the minimum of 500–1,000 which are immunogenic under certain circumstances [1].

As pointed out by GILL [6] and by BOREK [1], all of the states of organization of proteins and polypeptides provide structures that may be involved in the specificity of their antigenic determinants. Although the determinant site of β-lactoglobulin is not known, it is possible that it in-

volves not only primary and secondary structures but also tertiary and quaternary structures. It is recognized that the new antigen determinants in β-lactoglobulin may be sterically hidden, or if not so buried, they may be prevented from acting as immunogens by repression due to competition of the dominating determinant of intact β-lactoglobulin. Because of the lower molecular weights of the new antigens as compared with that of β-lactoglobulin and the denaturation of β-lactoglobulin that may have occurred in generation of the new antigens, it is speculated that the new antigen determinants are dependent more on their primary structures although newly formed secondary structures might also be involved.

The dialysate new antigens do not elicit precipitating antibodies in rabbits, whereas the six nondialysate new antigens (E1–E6) do elicit precipitating antibodies in rabbits [16]. A possible reason for this difference is the lower molecular weight of the dialysate new antigens or a simpler state of organization as compared with E1–E6.

It has been postulated that over 100 new antigens may be generated by pepsin digestion of milk proteins [16]. Although the clinical significance of these new antigens is not known, it is possible that one or more of them may elicit the IgE type antibodies usually associated with allergenicity. It is recognized that the several minor components detectable by disc electrophoresis of some dialysate subfractions may also have new antigen properties, a situation which could further complicate an already complex state. Answers to these questions must await future studies.

References

1 Borek, F.: Molecular size and shape of antigens; in Borek Immunogenicity (North-Holland, Amsterdam 1972).

2 Braunitzer, G.; Chen, R.; Schrank, B. und Stangl, A.: Automatische Sequenzanalyse eines Proteins (β-Lactoglobuline AB). Hoppe-Seylers Z. Physiol. Chem. *353:* 832–834 (1972).

3 Coulson, E. J.: The Schulz-Dale technique. J. Allergy *24:* 458–473 (1953).

4 Davis, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. *121:* 404–427 (1964).

5 Dunker, A. K. and Rueckert, R. R.: Observations on molecular weight determinations on polyacrylamide gel. J. biol. Chem. *244:* 5074–5080 (1969).

6 Gill, T. J., III.: The chemistry of antigens and its influence on immunogenicity; in Borek Immunogenicity (North-Holland, Amsterdam 1972).

7 Lyster, R. L. J.: Reviews of the progress of dairy science. Section C. Chemistry of milk proteins. J. Dairy Res. *39:* 279–318 (1972).

8 McKENZIE, H. A.: Milk proteins; in ANFINSEN, ANSON, EDSALL and RICHARDS Protein Chem. vol. 22 (Academic Press, New York 1967).
9 McKENZIE, H. A.; RALSTON, G. B., and SHAW, D. C.: Location of sulfhydryl and disulfide groups in bovine lactoglobulins and effect of urea. Biochemistry *11:* 4539–4547 (1972).
10 MOORE, S. and STEIN, W. H.: Chromatographic determination of amino acids by use of automatic recording equipment; in COLOWICK and KAPLAN Methods in enzymology, VI (Academic Press, New York 1963).
11 OBERMAYER, F. und PICK, E. P.: Über die chemischen Grundlagen der Arteigenschaften der Eiweisskörper. Bildung von Immunprazipitinen durch chemisch veränderte Eiweisskörper. Wien. klin. Wschr. *19:* 327 (1906).
12 SANGER, F. and THOMPSON, E. O. P.: Halogenation of tyrosine during acid hydrolysis. Biochem. biophys. Acta *71:* 468–471 (1963).
13 SPIES, J. R.: Determination of tryptophan in proteins. Analyt. Chem. *39:* 1412–1416 (1967).
14 SPIES, J. R. and CHAMBERS, D. C.: Chemical determination of tryptophan in proteins. Analyt. Chem. *21*: 1249–1266 (1949).
15 SPIES, J. R.; STEVAN, M. A.; STEIN, W. J., and COULSON, E. J.: The chemistry of allergens. XX. New antigens generated by pepsin hydrolysis of bovine milk proteins. J. Allergy *45:* 208–219 (1970).
16 SPIES, J. R.; STEVAN, M. A., and STEIN, W. J.: The chemistry of allergens. XXI. Eight new antigens generated by successive pepsin hydrolyses of bovine β-lactoglobulin. J. Allergy and Clin. Immunol. *50:* 82–91 (1972).
17 SPIES, J. R.; STEVAN, M. A., and STEIN, W. J.: A method for estimation of the relative antigenic potencies of preparations containing common new antigens derived from a precursor protein (β-lactoglobulin). J. Immunol. Meth. *2:* 35–43 (1972).
18 WEBER, K. and OSBORN, M.: Reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide-gel electrophoresis. J. biol. Chem. *244:* 4406–4412 (1969).